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Differential expression of Cux-1 and p21 in polycystic kidneys from Pkd1 null and cpk mice

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Background. Cux-1 is a murine homeodomain protein that functions as a cell cycle–dependent transcriptional repressor in proliferating cells. Targets of Cux-1 repression include the cyclin kinase inhibitors p21 and p27. In the kidney, Cux-1 is spatially and temporally regulated, and ectopic expression of Cux-1 in transgenic mice results in renal hyperplasia. Previously, we observed that Cux-1 is deregulated in cystic kidneys from cpk mice. Recent studies have suggested a role for the cyclin kinase inhibitor p21 in the development of polycystic kidney disease (PKD) in mice lacking PKD1.

Methods. Since p21 is a target of transcriptional repression by Cux-1, we compared the expression of Cux-1 and p21 in kidneys from Pkd1 null and cpk mice by immunohistochemistry and Western blotting. We also evaluated apoptosis and the expression of the cyclin kinase inhibitor p27 in Pkd1 null and cpk mice by terminal deoxynucleotidal transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining, immunohistochemistry, and Western blotting.

Results. In both early and late embryonic kidneys from Pkd1 null mice, Cux-1 was highly and ectopically expressed in normal-appearing tubule epithelium, interstitial cells, and in the epithelial cells lining the cysts, where it colocalized with proliferating cell nuclear antigen (PCNA). Increased Cux-1 expression in Pkd1 null kidneys was also associated with a decrease in p27 expression at late stages of cystogenesis. In cpk kidneys, Cux-1 was not up-regulated until late stages of cyst development. Moreover, in contrast to Pkd1 null kidneys, p21 and p27 were highly expressed in cpk kidneys. In late stages of cystogenesis, Cux-1 and p21 colocalized in cyst lining cells, which also showed a high incidence of apoptosis.

Conclusion. These results suggest that cyst development in Pkd1 null mice and cpk mice proceeds through different mechanisms. In Pkd1 null mice, ectopic expression of Cux-1 is associated with increased cell proliferation. In contrast, in cpk mice, ectopic expression of Cux-1 is associated with apoptosis.

Polycystic kidney disease (PKD) is a term applied to a group of inherited disorders characterized by the presence of cysts in the kidney. However, multiple organs are usually affected. Renal pathologies found in essentially all forms of PKD include increased fluid secretion, matrix remodeling, cellular proliferation, and apoptosis, with an altered differentiation of the epithelial cells lining the renal cysts [1–3]. PKD represents conditions that are inherited as either autosomal-dominant or autosomal-recessive traits. Autosomal-dominant PKD (ADPKD) occurs in 1 in 500 to 1 in 1000 individuals, primarily as a result of mutations in one of two genes, *PKD1* or *PKD2* [4–7]. The *PKD1* and *PKD2* genes encode polycystin-1 and polycystin-2, respectively. Mutations in *PKD1* are responsible for 85% of ADPKD, while mutations in *PKD2* are responsible for 15%. Autosomal-recessive PKD (ARPKD) is the more rapidly progressive of these conditions, which can lead to uremia in infants, is comparatively less frequent (1 in 6000 to 1 in 40,000 live births), and results from mutations in a single gene *PKHD1* [8, 9]. The protein encoded by *PKHD1* has been named polyductin or fibrocystin.

In recent years, the identification of the pathways disrupted by mutations in the polycystins, and leading to PKD, has been the focus of intense examination. Several lines of evidence suggest that dysregulation of epithelial cell growth is a key step in the process. The cystic epithelium has a high mitotic rate in vivo as measured by increased levels of expression of markers of proliferation. In the mouse, several distinct recessively acting mutations cause PKD phenotypes that mimic human disease [10]. Among these models, the congenital polycystic kidney (cpk) mutation is the most extensively characterized [11].

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The gene mutated in cpk mice has recently been identified and found to encode a protein product named cystin [12]. This protein is found in the primary cilium of epithelial cells, suggesting that ciliary dysfunction results in the PKD phenotype in cpk mice. In addition, *PKD1* and *PKD2* null mice [13–17] both produce cysts, and also localize to the primary cilium of epithelial cells [18]. These observations suggest that these genes may be involved in a common developmental cascade that is disrupted in PKD. Recently, the disruption of the gene encoding the KIF3A subunit of kinesin II specifically in the kidney, using the Cre-lox system, has linked cilia to cystogenesis [19]. Kinesin II is required for the anterograde movement of material in the cilia and loss of KIF3A results in the absence of tubulin in the cilia.

Cux-1 is a murine homeobox gene that is structurally related to the *Drosophila cut* gene. During normal kidney development, Cux-1 is highly and transiently expressed, with highest expression restricted to the nephrogenic zone, in a pattern similar to markers for cell proliferation [20]. Previous studies have demonstrated a role for Cux-1 in cell cycle regulation by repression of the cyclin kinase inhibitor p21 in G_1 to S transition in cultured cells [21]. In recent studies we showed that Cux-1 also represses expression of the cyclin kinase inhibitor p27, and that transgenic mice constitutively expressing Cux-1 develop multiorgan hyperplasia [22], phenocopying p27 null mice [23–25]. During normal kidney development, p27 is not expressed in the nephrogenic zone, but is upregulated in maturing glomeruli and tubules following down-regulation of Cux-1 [26]. Thus, Cux-1 functions to promote cell proliferation during early stages of nephrogenesis through repression of p27 gene expression.

Recent studies have demonstrated a role for polycystin-1 in cell cycle regulation. Bhunia, et al [27] showed that polycystin-1 induces expression of the cyclin kinase inhibitor p21 through activation of the JAK-STAT signaling pathway in a process requiring polycystin-2. Accordingly, loss of polycystin-1/2 function would result in down regulation of p21 expression and increased cell proliferation. Previously, we observed that Cux-1 is ectopically expressed in cystic kidneys from cpk mice [20]. Since p21 is a target of transcriptional repression by Cux-1, the loss of p21 expression may be a common mechanism for cystogenesis. To begin to address this possibility, we evaluated p21 and Cux-1 expression in Pkd1 null and cpk kidneys.

METHODS

Animals

Colonies of C57BL/6J cpk [11] and BALB/c-Pkd1 null [28] mice were maintained by breeding heterozygotes at the University of Kansas Medical Center. Genotyping of Pkd1 null mice was by Southern blot analysis of tail DNA using published procedures [28]. Kidneys from 7-, 9-, and 20-day-old normal and cystic cpk mice were perfusion-fixed, while kidneys from embryonic day 15 (E15) and 19 (E19) normal and cystic null mice were immersion-fixed, with 4% paraformaldehyde and paraffin-embedded. Normal mice included both wild-type and heterozygous genotypes. Animal treatment was in accord with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Immunofluorescence

Whole embryos or isolated metanephroi were immersion fixed in 4% paraformaldehyde and embedded in paraffin. Five micrometer thick tissue sections were deparaffinized with xylene and rehydrated with graded ethanols. To obtain adequate signal, the slides were treated with antigen unmasking solution (Vector, Burlingame, CA, USA) according to manufacturer's protocol, or slides were treated for 10 minutes with trypsin (1 mg/mL) at 37°C. To block endogenous auto fluorescence sections were incubated with 1 mol/L NH_4Cl for 30 minutes. Sections were washed in phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST), blocked in 10% BSA at room temperature for 1 hour, and incubated with antibodies diluted in PBS with 3% BSA for 1 hour at room temperature. Antibody dilutions were 1:50 for CCAAT displacement protein (Cux-1) (CDP) antibody, 1:3000 for proliferating cell nuclear antigen (PCNA) antibody, 1:100 for p21 antibody, 1:100 for p27 antibody, in 2% blocking serum in PBS. Slides were incubated at room temperature with 100 μL of primary antibody in a humid chamber and then washed four times in PBST. For Cux-1 and p21 double-labeling experiments sections were washed with PBST and incubated simultaneously with biotin-conjugated horse antirabbit (Vector) and Texas-Red-conjugated donkey antigoat secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were then washed with PBST and incubated with fluorescein isothiocyanate (FITC)-conjugated avidin (Vector). For Cux-1 and PCNA double-labeling experiments, sections were washed with PBST and incubated simultaneously with biotin-conjugated horse antirabbit and Texas-Red-conjugated rabbit antimouse secondary antibodies (Vector). Sections were then washed with PBST and incubated with FITC-conjugated avidin (Vector). Sections were then washed, mounted with Vectashield (Vector), and viewed with a fluorescence microscope. Images were captured with an Optronics Magnafire digital camera (Goleta, CA, USA).

Western blot analysis

Nuclear extracts (30 μg) were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, electrophoresed on 4% to 15% gradient polyacrylamide gels, and transferred to nitrocellulose filters as described previously [29]. The

immunoblot was blocked in 5% nonfat dry milk in PBST for 1 hour at room temperature. Mouse anti-p21 (Santa Cruz Biotechnology) antibody was added at a dilution of 1:100. After overnight at 4°C, filters were washed three times at room temperature with PBST, and incubated at room temperature for 1 hour with peroxidase-conjugated antimouse IgG (1:10,000 dilution) (Sigma Chemical Co., St. Louis, MO, USA). Following three additional washes with PBST, bound antibody was detected by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate) (Pierce, Rockford, IL, USA) according to manufacturer's instructions, followed by exposure to x-ray film for 1 minute.

Antibodies

Commercial reagents used were rabbit anti-CDP (Cux-1) (Santa Cruz Biotechnology, # sc-13024); goat anti-p21 (Santa Cruz Biotechnology); mouse anti-p21 (Santa Cruz Biotechnology); rabbit anti-p27 (Santa Cruz Biotechnology); and mouse anti-PCNA (Sigma Chemical Co., # P-8825).

TUNEL assay

Sections were processed for terminal deoxynucleotidal transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) with the ApopTag Red In Situ Apoptosis Detection Kit (Intergen Co., Purchase, NY, USA) according to manufacturer's instructions. Sections were counterstained with DAPI, cover-slipped, and visualized on a fluorescence microscope. Images were captured with an Optronics Magnafire digital camera. For Cux-1 and TUNEL double-labeling experiments, sections were incubated first with Cux-1 antibody followed by FITC-conjugated goat antirabbit secondary antibodies. TUNEL was then performed on the same sections, according to manufacturer's directions. Sections were counterstained with DAPI, cover-slipped, and visualized on a fluorescence microscope. Images were captured with an Optronics Magnafire digital camera.

RESULTS

We have recently identified a role for Cux-1 in regulating the cell cycle in kidney development by controlling the expression of the cyclin kinase inhibitor p27 [22]. Together with the finding that Cux-1 represses p21 in a cell cycle–dependent manner [21], these studies support a general role for Cux-1 in growth regulation, and suggest that ectopic expression of Cux-1 could be a determinant in disease progression. This is supported by the observation that ectopic expression of Cux-1 in transgenic mice results in multiorgan hyperplasia [22], glomerulosclerosis [30], and hepatic tumors (submitted manuscript).

Recent studies have shown that polycystin-1 regulates p21 gene expression by signaling through the JAK-STAT pathway [27]. Accordingly, p21 expression was lost in PKD1 null mice [27]. Together with the observation that Cux-1 is ectopically expressed in cystic kidneys isolated from cpk mice, we considered the possibility that the down-regulation of p21 was a common mechanism in cystogenesis and that ectopic expression of Cux-1 might be mediating this process. To begin to address these possibilities, we began an expression analysis of p21 and Cux-1 in cystic kidneys from cpk and Pkd1 null mice.

Cux-1 is ectopically expressed in Pkd1 null kidneys

To determine whether Cux-1 was ectopically expressed in Pkd1 null kidneys, we evaluated Cux-1 expression in kidneys isolated from early (E15) and late (E19) stages of cystic progression. During nephrogenesis, Cux-1 expression is restricted to the uninduced and condensing mesenchyme, and early nephric structures (comma and S-shaped bodies) found in the nephrogenic zone, with lower levels of expression observed in the medulla. This pattern of expression is highly similar to that of PCNA, indicating that Cux-1 expression corresponds to proliferating cells. In cytomegalovirus (CMV)/Cux-1 transgenic kidneys, the ectopic expression of Cux-1 in the tubules results in an increase in cell proliferation, and the ectopic expression of Cux-1 in mesangial cells results in an increase in PCNA staining. As shown in Figure 1A, Cux-1 is highly expressed in the nephrogenic zone of normal kidneys, but is sharply down-regulated following S-shaped body stage. In contrast, Cux-1 was highly and ectopically expressed in Pkd1 null kidneys, both in the cyst lining cells, and in normal appearing tubule epithelium (Fig. 1B). At both early (E15) and late (E19) stages of cystogenesis in Pkd1 null kidneys, we observed Cux-1 expression in the nephrogenic zone, in maturing tubules, and in the cells lining the cysts (Fig. 2B and J). In both stages, Cux-1 and PCNA were colocalized in the nephrogenic zone and in the cells lining the cysts, consistent with the previously described role for Cux-1 in regulating cell proliferation (Fig. 2D and L). However, in some of the maturing tubules, we observed ectopic expression of Cux-1, but not PCNA (Fig. 2H).

Expression of the cyclin kinase inhibitor p27 is reduced in Pkd1 null kidneys

We next evaluated the expression of p21 in early and late stage cystic kidneys from Pkd1 null mice. As previously described, p21 was not detected in kidneys from E15 Pkd1 null mice (data not shown), or in kidneys from E19 Pkd1 null mice (Fig. 3B). In addition to negatively regulating the expression of p21, we have recently shown that Cux-1 represses p27 gene expression. Moreover, in characterizing the CMV/Cux-1 transgenic mice, we observed

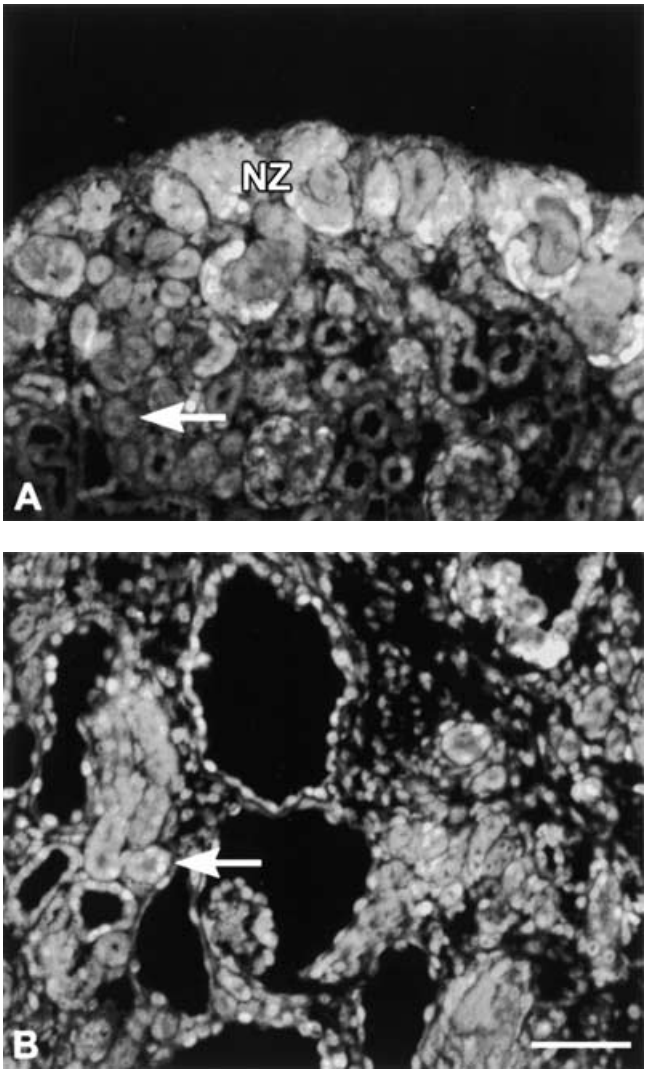

Fig. 1. Cux-1 is ectopically expressed in kidneys isolated from Pkd1 null mice. (*A*) Cux-1 expression is restricted to the nephrogenic zone (NZ) in late embryonic wild-type kidneys. (*B*) Kidneys isolated from embryonic day 19 Pkd1 null mice reveal ectopic expression of Cux-1 in both normal tubules (arrow) and in cells lining the cysts (original magnification 400×; bar, 50 μm).

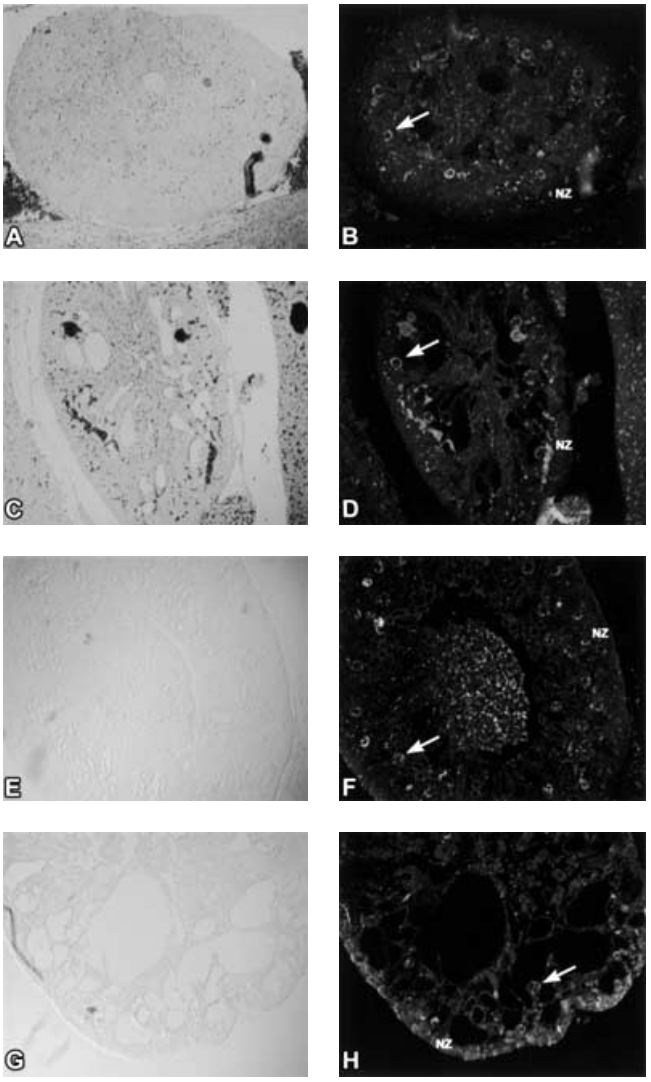

Fig. 4. Reduced expression of p27 in kidneys from E19 Pkd1 null mice. Embryonic day 15 (E15) (*A* to *D*) and E19 (*E* to *H*) kidneys isolated from wild-type (A, B, E, F) and Pkd1 null (C, D, G, H) mice were labeled with an antibody directed against p27. (B and F) p27 is not expressed in the nephrogenic zone of kidneys isolated from wild-type E19 mice, but is up-regulated in maturing glomeruli (arrow) and tubules. p27 is expressed at similar levels to wild-type in cystic kidneys isolated from E15 Pkd1 null mice (D), but p27 expression is reduced in maturing tubules of cystic kidneys isolated from E19 Pkd1 null mice. p27 expression was observed in maturing glomeruli of both wild-type and cystic kidneys (arrows) (original magnification 100×; bar, 200 μm).

that p27 knockout mice have similar deregulated growth defects, and the ectopic expression of Cux-1 resulted in the loss of p27. In contrast, the p21 knockout mice do not exhibit growth defects [31]. Thus, one possibility is that the deregulated expression of Cux-1 in Pkd1 null kidneys could be repressing the expression of p27, leading to abnormal cell proliferation. To test this, we evaluated p27 expression in kidneys from E15 and E19 Pkd1 null and wild-type mice. During normal kidney development, p27 was highly expressed in maturing glomeruli and tubules, but was not expressed in the nephrogenic zone, consistent with repression by Cux-1 in proliferating cells (Fig. 4B and F). At E15, there was not an obvious difference in p27 expression between the wild-type and Pkd1 null kidneys (Fig. 4B and D). In contrast, p27 expression was reduced in the tubules and cysts of kidneys from E19 Pkd1 null mice, although some expression was observed in glomeruli (Fig. 4H).

Cux-1 and PCNA are not colocalized in cystic kidneys from cpk mice

Previously, we had examined Cux-1 mRNA expression in kidneys from 21-day-old cystic cpk mice, when cystogenesis is well advanced, and primarily of collecting duct

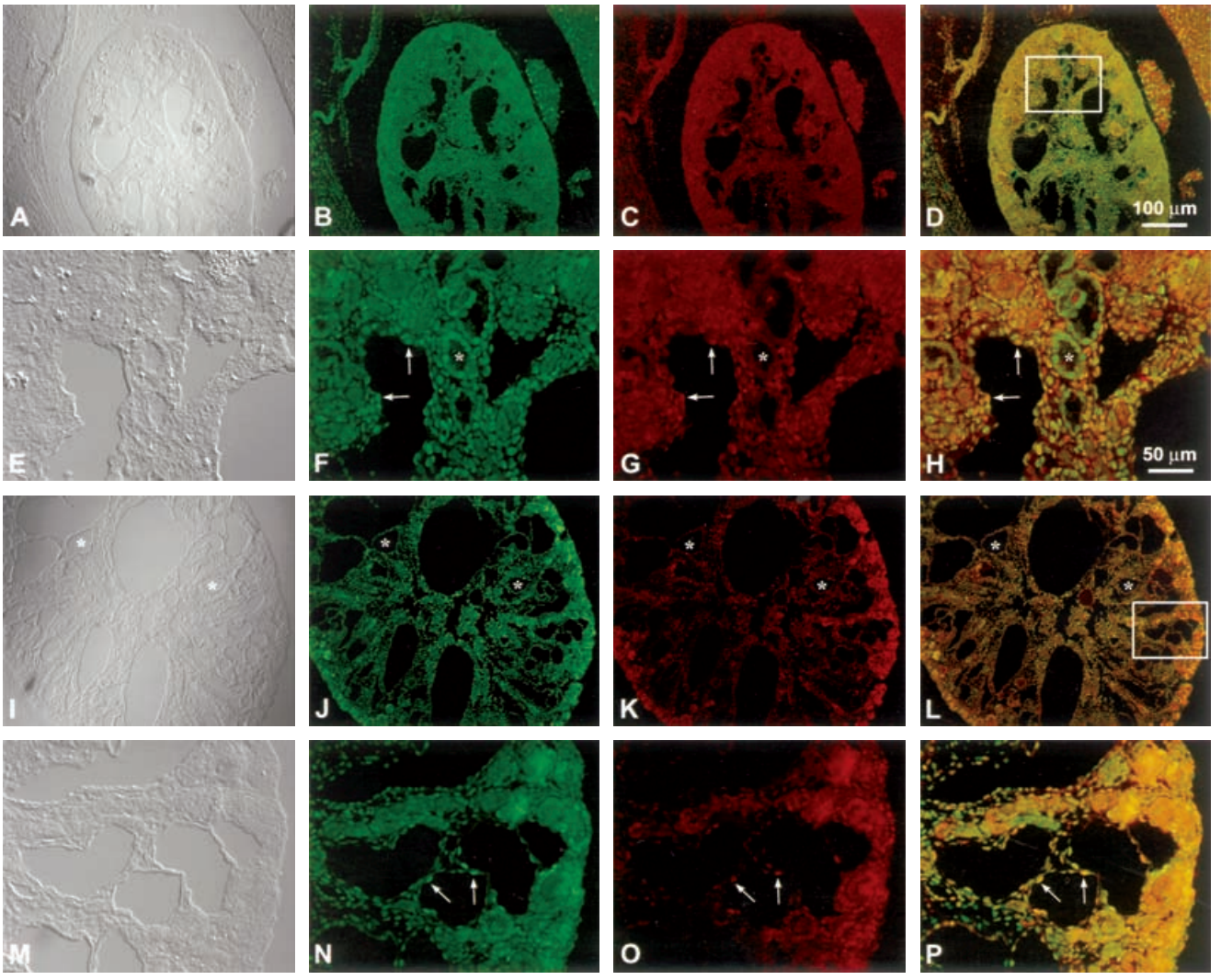

Fig. 2. Coexpression of Cux-1 and proliferating cell nuclear antigen (PCNA) in the cysts of kidneys from Pkd1 null mice. Early- [embryonic day 15 (E15)] and late- (E19) stage polycystic kidneys were evaluated for expression of Cux-1 and PCNA. Low (*A* to *D*) and high (*E* to *H*) magnification images of kidneys isolated from E15 Pkd1 null mice following labeling for Cux-1 (B and F) and PCNA (C and G) expression. At early stages of cystogenesis (A to H), Cux-1 was ectopically expressed in maturing tubules and in cysts (B and F). PCNA labeling (C and G) showed proliferating cells lining the cysts, but not in all cells. (D) Merged image (B and C) [boxed region is shown in (H)]. (H) The merged Cux-1 and PCNA images showed colocalization in cyst lining cells (arrows), but not in some tubules that were positive for Cux-1 only (∗). (*I* to *P*) At late stages of cystogenesis, Cux-1 expression remained elevated in maturing tubules and cysts (J and N), and colocalized with PCNA throughout the cystic kidneys, both in the nephrogenic zone and in cells lining the cysts (∗ and arrows). Boxed region in (L) is shown at higher magnification in (P) [original magnification (A to D, I to L) 200×; bar, 100 μm; (E to H, M to P) 400×; bar, 50 μm].

origin [20]. For the present studies, we used antibodies to localize Cux-1 protein, beginning at postnatal day 7, when only a few cysts are present, and cysts of both proximal tubule and collecting duct origin are present. In cystic kidneys from 7-day-old mice, Cux-1 was highly expressed in the nephrogenic zone, where it colocalized with PCNA, as expected (Fig. 5B to D). In the medulla, we observed increased PCNA expression in the cyst lining epithelium (Fig. 5G); however, in contrast to the early staged Pkd1 null kidneys, Cux-1 was not ectopically expressed in the cysts and did not colocalize with PCNA in these cells (Fig. 5H). The finding that Cux-1 was not ectopically expressed in early cystic kidneys, prompted us to reexamine its expression at later stages of cystogenesis. Similar to our previous results [20], we found that Cux-1 was indeed ectopically expressed in the cyst lining cells when cystogenesis is well advanced (Fig. 5J and N). However, in contrast to the kidneys from E19 Pkd1 null, we found that the ectopic expression of Cux-1 did not colocalize with PCNA in many of the cyst lining cells (Fig. 5P).

Ectopic expression of p21 in cystic kidneys from cpk mice

We next evaluated the expression of p21 in early and late stage cystic kidneys from cpk mice. While p21 is normally down-regulated by this stage of kidney development (Fig. 6C) [32], to our surprise, p21 was ectopically

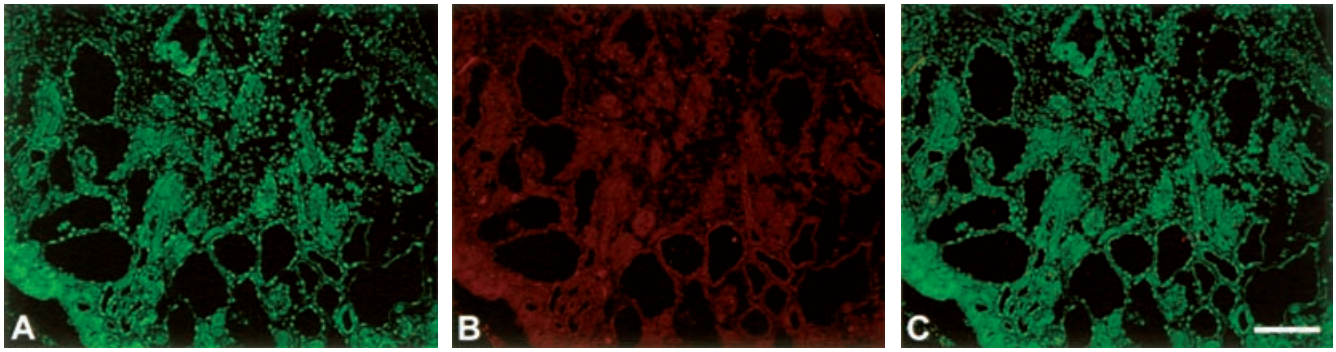

Fig. 3. Reduced expression of p21 in kidneys from Pkd1 null mice. Kidneys isolate from embryonic day 19 (E19) Pkd1 null mice were labeled with antibodies directed against Cux-1 and p21. (*A*) Cux-1 is ectopically expressed in cyst lining cells. (*B*) p21 is not expressed in the in the tubules or cysts of Pkd1 null kidneys. (*C*) Since p21 is not expressed in Pkd1 null kidneys, there is no overlap in the merged image (original magnification 200×; bar, 100 μm).

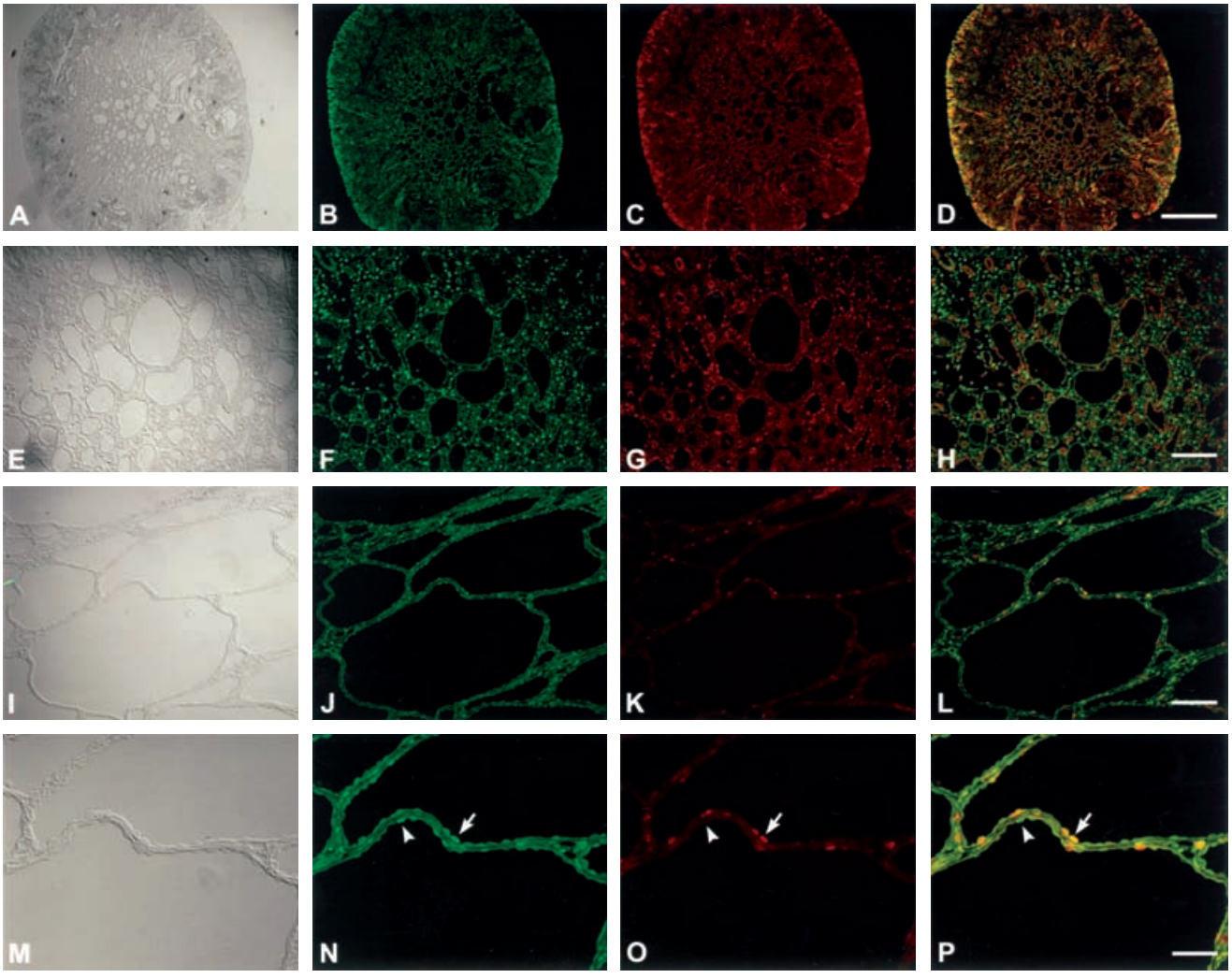

Fig. 5. Differential expression of Cux-1 and proliferating cell nuclear antigen (PCNA) in the cysts of 7-day-old and 20-day-old cpk kidneys. Early- (7-day-old) and late- (20-day-old) stage polycystic kidneys from cpk mice were labeled with antibodies for Cux-1 (B, F, J, N) and PCNA (C, G, K, O). (*A* to *H*) In kidneys from 7-day-old cpk mice, Cux-1 and PCNA colocalize in the nephrogenic zone (D), but not in the medulla where cysts are forming (H). Cells lining the cysts are PCNA positive (G), while Cux-1 expression is restricted to interstitial cells (F). (*I* to *P*) In kidneys from 20-day-old cpk mice, Cux-1 is ectopically expressed in cells lining the cysts (J, arrow) and (N, arrowhead). While some cells lining the cysts are PCNA positive (O, arrow), most are not. Merged image (P) shows that Cux-1 is expressed in most of the cyst lining cells, but only a few are also positive for PCNA (arrow) [original magnification (A to D) 50×; bar, 500 μm; (E to L) 200×; bar, 100 μm; (M to P) 400×; bar, 50 μm).

expressed both in cyst lining epithelium and in the normal appearing tubules of the cystic kidneys from 7- and 9-day-old mice (Fig. 6G and K). Double-labeling showed that Cux-1 and p21 did not colocalize in any of the cells (Fig. 6H and L). Rather Cux-1 expression was restricted to the interstitial cells, similar to the expression in wild-type kidneys (compare Fig. 6B with F and J). In kidneys from 20-day old cpk mice, we found a colocalization of Cux-1 and p21, both in normal appearing tubules and in some of the cyst lining cells (Fig. 7C). To confirm the increase in p21 expression in the cystic kidneys, we evaluated p21 protein expression by Western blot analysis. Figure 8A shows that p21 protein was increased in cystic kidneys from 16- and 21-day old-mice compared to the age-matched normal kidneys. We next evaluated the expression of p27 in cpk kidneys by Western blot analysis. Similar to p21, p27 was also ectopically expressed in kidneys from cpk mice (Fig. 8B).

There is accumulating evidence that the processes of cell cycle progression and apoptosis are coupled, through the utilization of a common set of proteins, including the cyclin kinase inhibitors. The colocalization of Cux-1 and p21 in cystic kidneys, together with the differential localization of Cux-1 and PCNA, suggested that Cux-1 was uncoupled from the role of repressing p21 to promote cell proliferation. One possibility is that the apparently disparate expression of Cux-1 and p21 might result in an apoptotic signal. To test this, we labeled cystic kidneys for apoptosis using the TUNEL assay. We observed a high incidence of apoptosis in late-stage cpk kidneys, primarily in the cells lining the cysts (Fig. 9K). In contrast, many fewer TUNEL-positive cells were observed in early-stage cpk kidneys, when Cux-1 and p21 were not coexpressed (Fig. 9H). We also observed some TUNEL-positive cells in Pkd1null kidneys, both at early and late stages of cystogenesis (Fig. 9B and E). However, the number of apoptotic cells did not increase dramatically between early and late stages of cystogenesis, and there were many fewer apoptotic cells in the cysts in the late-stage Pkd1 null kidney, compared to the late-stage cpk kidney. To determine whether the ectopic expression of Cux-1 was associated with apoptosis, we performed double-labeling of Cux-1 with TUNEL on kidney sections from 20-day-old cpk mice. This showed that many of the many of the Cux-1–positive cyst lining cells were undergoing apoptosis (Fig. 10).

DISCUSSION

In previous studies we demonstrated that *Cux-1* expression in the developing kidney is inversely related to degree of cellular differentiation, and that *Cux-1* expression is deregulated in the cpk mouse model of PKD [20]. To test the hypothesis that *Cux-1* must be down-regulated for normal kidney development, we generated transgenic mice constitutively expressing Cux-1 using the CMV promoter. These mice developed multiorgan hyperplasia, and in the kidney, this resulted from the down regulation of the cyclin kinase inhibitor p27. Promoter reporter assays revealed that Cux-1 is a repressor of p27 gene expression. Together with previous studies documenting repression of the cyclin kinase inhibitor p21 by Cux-1, these results indicate a role for Cux-1 as a cell cycle–dependent transcription factor in proliferating cells.

Recently, a role for polycystin-1 in regulating the cell cycle has been established. Bhunia et al [27] showed that polycystin-1, in association with polycystin-2, activates the JAK-STAT pathway, thereby up-regulating p21 and inducing cell cycle arrest in G_0/G_1. Furthermore, mouse embryos lacking PKD1 had defective STAT1 phosphorylation and p21 induction. Together, these results suggested a mechanism by which mutations of either polycystin-1 or -2 can result in deregulated growth. Since p21 is a transcriptional target of repression by Cux-1, we hypothesized that a reduction in p21 might be a common mechanism of deregulated cell proliferation in cystogenesis, and this might be mediated by the ectopic expression of Cux-1. To begin to address these possibilities, we analyzed the expression of p21 and Cux-1 in cystic kidneys from cpk and Pkd1 null mice.

In cystic kidneys isolated from Pkd1 null embryos Cux-1 was highly and ectopically expressed in both cystic and normal tubules. Moreover, this ectopic expression of Cux-1 was associated with increased cell proliferation, as Cux-1 and PCNA were colocalized in cells lining the cysts, as well as in noncystic tubules. We did not detect p21 in Pkd1 null kidneys, as has been previously described [27]. Although the regulation of p21 by polycystin-1 and the loss of p21 in Pkd1 null mice have provided a direct link between polycystin-1 and cell cycle regulation, there may be other factors contributing to the increased proliferation observed in PKD. This is evidenced by the fact that p21 null mice do not exhibit a cell proliferation defect [31]. p27 null mice, however, do exhibit cell proliferation defects leading to multiorgan hyperplasia [23–25]. We have recently identified a potential regulatory loop between Cux-1 and p27. Cux-1 transgenic mice develop multiorgan hyperplasia, phenocopying p27 null mice, and transfection assays showed that Cux-1 represses p27 promoter activity [22]. We therefore examined p27 expression in cystic kidneys from Pkd1 null mice and found that p27 expression was reduced compared to wild-type kidneys. It is, therefore, plausible that another mechanism of deregulated cell proliferation in polycystic kidney disease is repression of p27 by ectopically expressed Cux-1. Together with the changes observed in the Cux-1 transgenic mice, these results suggest a more direct link to abnormal cell proliferation. Moreover, since both p21 and p27 are targets of Cux-1, there may be active repression of p21 by

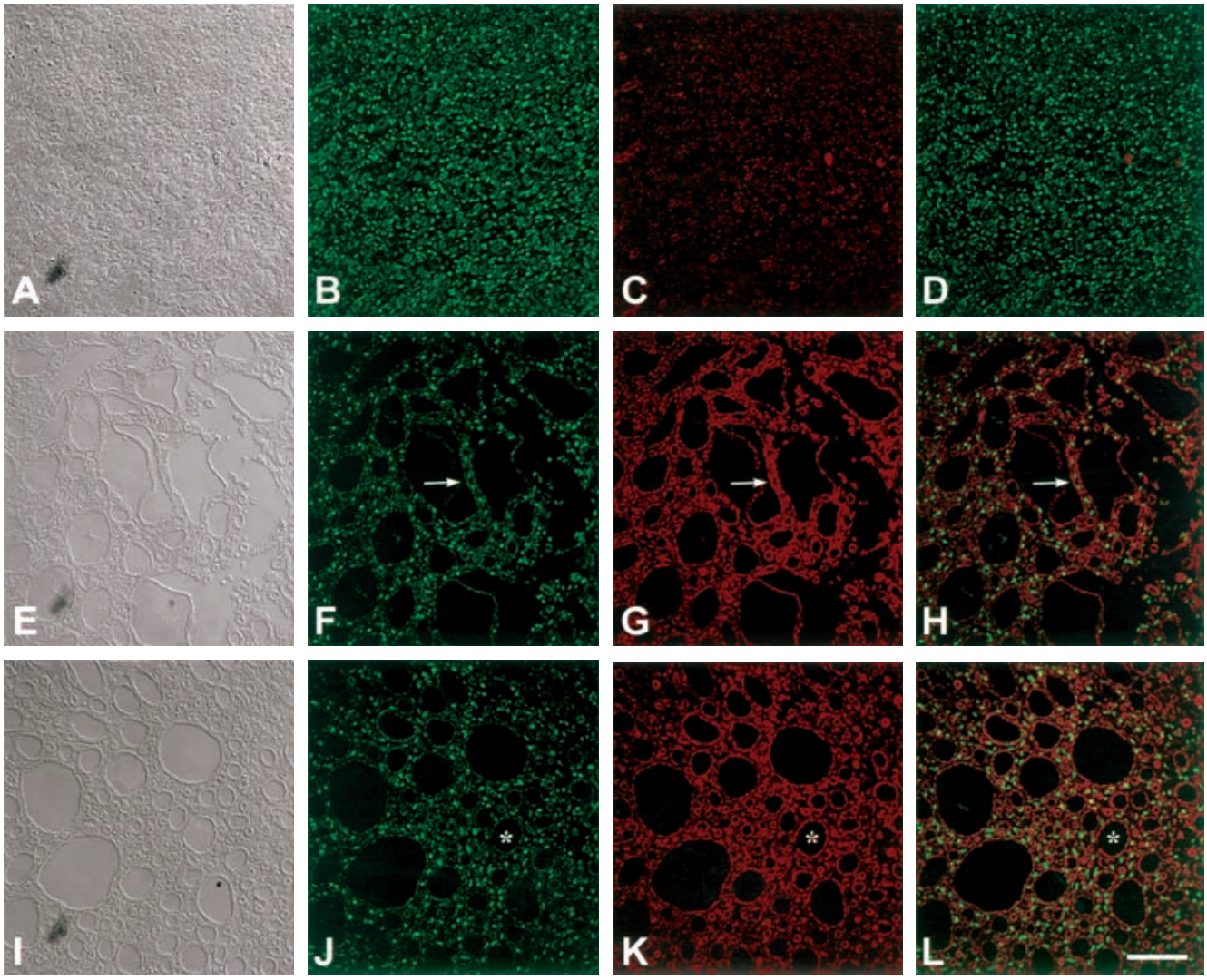

Fig. 6. p21 is ectopically expressed in the cysts of kidneys from 7- and 9-day-old cpk mice. (*A* to *D*) Kidneys from 7-day-old wild-type mice were labeled with antibodies for Cux-1 (B) and p21 (C). Cux-1 is expressed primarily in interstitial cells, while p21 expression is not detected. (*E* to *H*) Kidneys from 7-day-old cpk mice were labeled with antibodies for Cux-1 (F) and p21 (G). Cux-1 is expressed primarily in interstitial cells. In contrast to wild-type, p21 expression is up-regulated in cells lining the cysts (G). Cux-1 and p21 do not colocalize (H). (*I* to *L*) Cux-1 and p21 expression in kidneys from 9-day-old cpk mice localizes similarly to kidney from 7-day-old cpk mice. Cux-1 and p21 do not colocalize (L) (original magnification 200×; bar, 100 μm).

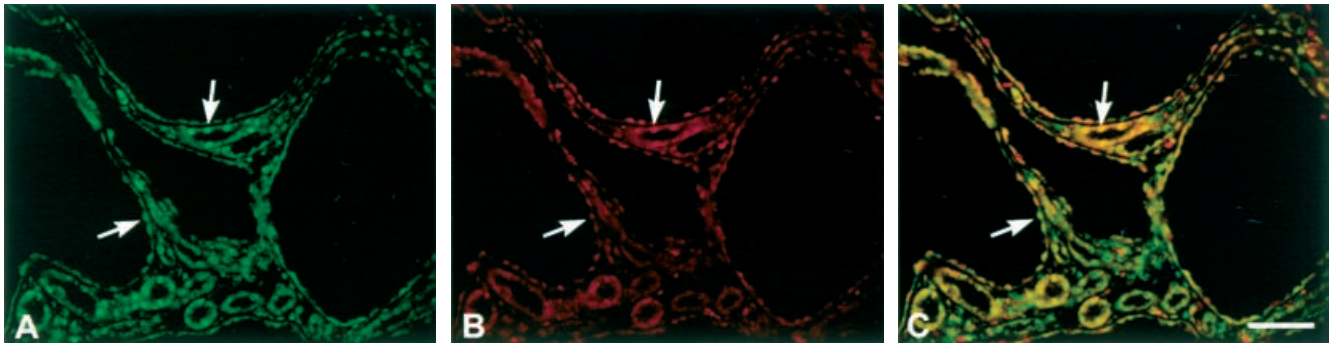

Fig. 7. Colocalization of Cux-1 and p21 in kidneys from 20-day-old cpk mice. Kidneys from 20-day-old cpk mice were labeled with antibodies for Cux-1 and p21. Cux-1 (*A*) and p21 (*B*) are ectopically expressed in both cyst lining cells and in normal appearing tubule epithelium (arrow). In some tubules, Cux-1 and p21 expression colocalize (*C*) (original magnification 400×; bar, 50 μm).

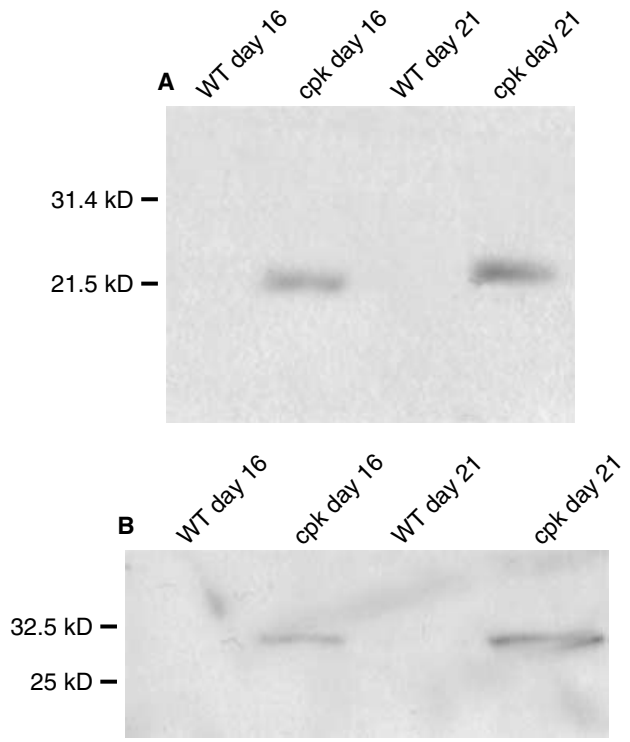

Fig. 8. Ectopic expression of p21 and p27 protein in cpk kidneys. Thirty micrograms of nuclear extract prepared from kidneys from 16- and 21-day-old wild type (WT) and cpk mice was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. (*A*) The presence of p21 protein was detected by using a monoclonal antibody to p21. p21 was detected at high levels in cpk kidneys, but not in age-matched wild-type kidneys. (*B*) Similarly, p27 was detected at high levels in cpk kidneys, but not in age-matched wild-type kidneys. Equal loading of protein was confirmed by Ponceau S staining. Molecular weight standards are shown on right.

the ectopically expressed Cux-1contributing to the loss of p21 gene activation in Pkd1 null mice.

In contrast to Pkd1 null mice, our results showed that p21 was ectopically expressed in the cystic kidneys from cpk mice, primarily in the cells lining the cysts. In addition, we observed an increased expression of p27 in cpk kidneys. Furthermore, during the early stages of cystogenesis, Cux-1 was not ectopically expressed. Cux-1 was localized to interstitial cells in both wild-type and cpk cystic kidneys, but not in the cystic cells. This appeared to be in contrast to our previous studies showing ectopic expression of Cux-1 in cystic kidneys from 21-day-old cpk mice. However, when we examined Cux-1 expression in kidneys from 20-day-old cpk mice, we observed that Cux-1 was indeed ectopically expressed in cystic and noncystic tubules. In addition, p21 was also ectopically expressed at this stage, and colocalized with Cux-1 in many of the cells. We also observed a high incidence of apoptosis in the cells lining the cysts, as has been observed by others [33–35]. There is accumulating evidence that the processes of cell cycle progression and apoptosis are coupled, through the utilization of a common set of proteins, including p21. One possibility is that in late stages of cystic disease in the cpk kidney, Cux-1 was uncoupled from the role of repressing p21 and promoting cell proliferation, to a role in apoptosis. This is supported by the observation that many of the cells ectopically expressing Cux-1 were also undergoing apoptosis. One possibility is that the co-expression of Cux-1 and p21 results in the direct activation of the apoptotic pathway. Alternatively, apoptosis may be secondary to these cells receiving multiple contradictory signals. Since we were unable to definitively show that the cells expressing both Cux-1 and p21 are also apoptotic, by simultaneously labeling for Cux-1, p21, and TUNEL, the mechanism remains unclear. However, the high levels of apoptosis were not observed at the early stages of cystogenesis in the cpk kidneys, even though p21 was highly expressed in the cyst lining cells. Nor were high levels of apoptosis observed in Pkd1 null kidneys, even though Cux-1 was highly expressed in the cyst lining cells. Taken together, these results suggest that the ectopic expression of both Cux-1 and p21 is associated with the increase in apoptosis observed in the 20-day-old cpk kidneys. Recent studies have shown that polycystin-1 is overexpressed in cpk kidneys [36]. Thus, it is possible that this increase in polycystin-1 results in the ectopic expression of p21 in the cpk kidneys.

While ectopic expression of Cux-1 correlated with increased cell proliferation in Pkd1 null kidneys, this was not the case in cpk kidneys. Cux-1 and PCNA colocalized in the nephrogenic zone, but not in the cyst lining cells of the kidneys from 7- or 9-day-old cpk mice. In addition, the cyclin kinase inhibitors p21 and p27 were up-regulated in late stage cpk kidneys, but were down-regulated in late-stage PKD1 null kidneys. One possible explanation for these expression differences is that cystogenesis in cpk and Pkd1 null kidneys proceed through different mechanisms. In the case of the Pkd1 null mice, the cysts develop concurrently with nephron formation. Thus, the ectopic expression of Cux-1 likely results in an exaggerated, normal function of Cux-1. During normal kidney development, Cux-1 functions to repress p27 in the nephrogenic zone allowing cells to proliferate. Accordingly, in the Pkd1 null cystic kidneys, the ectopic expression of Cux-1 in maturing and cystic tubules would repress p27, promoting cell proliferation. This is supported by the colocalization of Cux-1 and PCNA in these cells. In the case of the cpk mice, gross cyst formation occurs primarily during postnatal renal maturation, at a time when Cux-1 is normally down-regulated, and after terminal differentiation. Moreover, Cux-1 is not ectopically expressed until the cysts are very large and encompass most of the kidney. Consequently, during the early stages of cystogenesis in the cpk mouse, cell proliferation is Cux-1 independent. At later stages of cystogenesis in the cpk mouse, Cux-1 expression colocalizes with p21, and appears to be related to

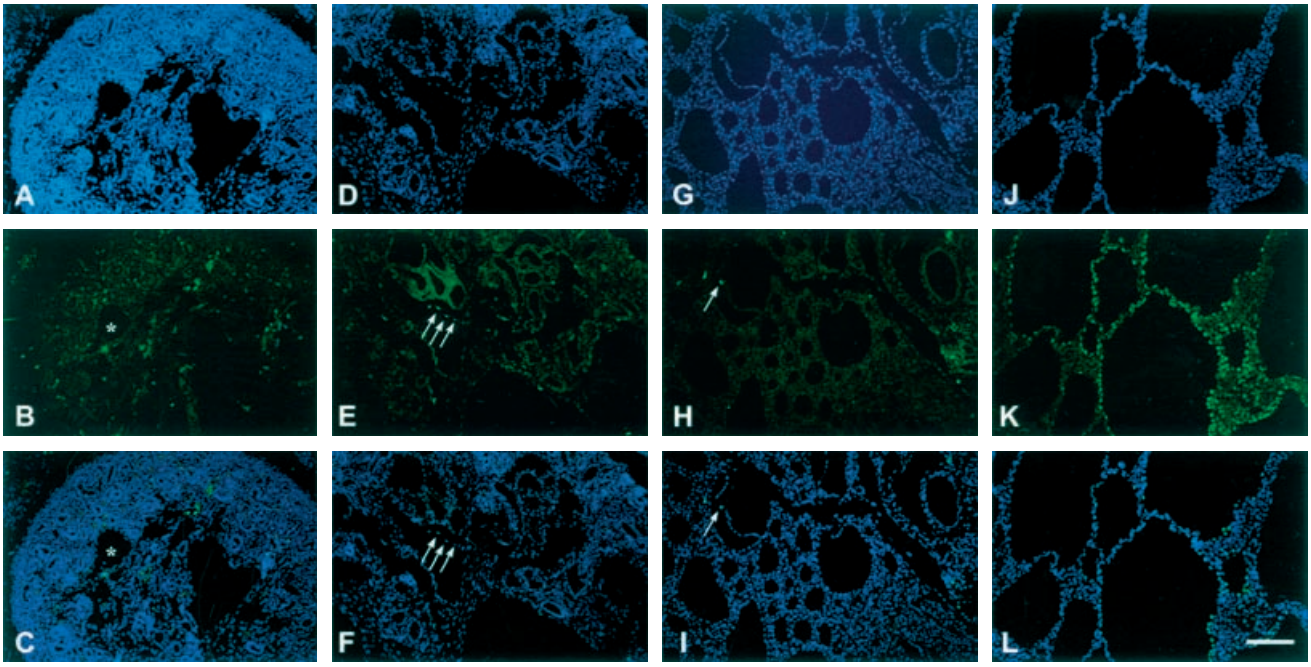

Fig. 9. Increased apoptosis in late stage cpk kidneys. (*A*) The terminal deoxynucleotidal transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) method was used to identify apoptotic cells in early (B and H) and late (E and K) stages of cystogenesis in kidneys from Pkd1 null (B and E) and cpk (H and K) mice. (*B*) Apoptotic cells were observed primarily in the nephrogenic zone of the kidneys from embryonic day 15 (E15) Pkd1 null mice, with few apoptotic cells in the cysts (∗). (*C*) The TUNEL staining was merged with the nuclear stain (DAPI) (A) to identify TUNEL-positive cells. (*D* to *F*) Few apoptotic cells are observed among the cyst lining cells in the kidneys from E19 Pkd1 null mice (arrow). (*G* to *I*) Similarly, there are few apoptotic cells found among the cyst lining cells in the kidneys from 7-day-old cpk mice (arrow). (*J* to *L*) In contrast, numerous TUNEL-positive cells are observed among the cells lining the cysts in the kidneys from 20-day-old cpk mice. The merged TUNEL and DAPI image (L) shows that TUNEL-positive staining corresponds to nuclei (original magnification 200×; bar, 100 μm).

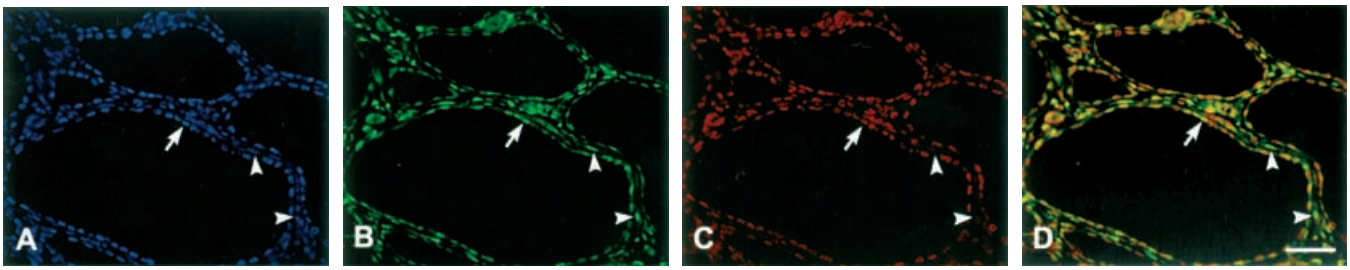

Fig. 10. Colocalization of Cux-1 and terminal deoxynucleotidal transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) cyst lining cells of late-stage cpk kidneys. (*A*) To determine whether Cux-1–positive cells were also apoptotic, sections of kidneys from 20-day-old cpk mice were labeled with antibodies directed against Cux-1, followed by TUNEL labeling. (*B*) Cux-1 was ectopically expressed in many of the cyst lining cells (arrows). (*C*) Apoptotic cells were observed among many of the cyst lining cells (arrows). (*D*) The merged image shows that most of the cyst lining cells were both Cux-1 and TUNEL-positive (arrow), while most of the interstitial cells only expressed Cux-1 (arrowheads) (original magnification 400×; bar, 50 μm).

increased levels of apoptosis and not proliferation. Taken together, these results suggest that in postnatal cystic disease, Cux-1 function is uncoupled from the normal regulation of p21 and p27 gene expression.

Recent studies demonstrate that the proteins encoded by the *PKD1* (polycystin-1), *PKD2* (polycystin-2), *Tg737* (polaris), and *cpk* (cystin) genes are colocalized in renal cilia [18]. These results suggest that disruption of the genes encoding these proteins may disrupt ciliary function to a greater or lesser extent, altering a common signaling pathway. One possibility is that Cux-1 expression and/or function, is regulated by this common signaling pathway, and levels of Cux-1 expression, and/or changes in function, may contribute to the severity of the cystic phenotype. Our results suggest that the role of Cux-1 as a transcriptional regulator of the cell cycle is retained in the Pkd1 null kidneys, but expression is altered. In contrast, in the cpk kidneys, this function of Cux-1 does not appear to play an important role, at least in the early stages of cystogenesis. Rather, the ectopic expression of Cux-1 is associated with apoptosis. It is unlikely that this simply results from increased expression of Cux-1 in cells following

terminal differentiation, since no increase in apoptosis was observed in the renal tubules of adult Cux-1 transgenic mice [22]. Moreover, the studies on the Cux-1 transgenic mice demonstrate that ectopic expression of Cux-1 alone is insufficient to cause cyst development. Rather, the levels of Cux-1 expression, or changes in Cux-1 function, may modify cyst progression. Future studies will be required to delineate the role of Cux-1 in cyst progression.

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REFERENCES

1. Grantham JJ: Mechanisms of progression in autosomal dominant polycystic kidney disease. *Kidney Int* (Suppl 63):S93–S97, 1997
2. Calvet JP, Grantham JJ: The genetics and physiology of polycystic kidney disease. *Semin Nephrol* 21:107–123, 2001
3. Igarashi P, Somlo S: Genetics and pathogenesis of polycystic kidney disease. *J Am Soc Nephrol* 13:2384–2398, 2002
4. The European Polycystic Kidney Disease Consortium: The polycystic kidney disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. *Cell* 77:881–894, 1994
5. The American PKD1 Consortium: Analysis of the genomic sequence for the autosomal dominant polycystic kidney disease (PDK1) gene predicts the presence of a leucine-rich repeat. *Hum Mol Genet* 4:575–582, 1995
6. The International Polycystic Kidney Disease Consortium: Polycystic kidney disease: The complete structure of the PKD1 gene and its protein. *Cell* 8:289–298, 1995
7. Mochizuki T, Wu G, Hayashi T, *et al*: PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. *Science* 272:1339–1342, 1996
8. Onuchic LF, Furu L, Nagasawa Y, *et al*: PKHD1, the polycystic kidney and hepatic disease 1 gene, encodes a novel large protein containing multiple immunoglobulin-like plexin transcription factor domain and parallel beta-helix 1 repeats. *Am J Hum Genet* 70:1305–1317, 2002
9. Ward CJ, Hogan MC, Rossetti S, *et al*: The gene mutated in autosomal recessive polycystic kidney disease encodes a large, receptor-like protein. *Nat Genet* 30:259–269, 2002
10. Schieren G, Pey R, Bach J, *et al*: Murine models of polycystic kidney disease. *Nephrol Dial Transplant* 11 (Suppl 6):38–45, 1996
11. Gattone VH 2nd, Calvet JP, Cowley BD Jr., *et al*: Autosomal recessive polycystic kidney disease in a murine model. A gross and microscopic description. *Lab Invest* 59:231–238, 1988
12. Hou X, Mrug M, Yoder BK, *et al*: Cystin, a novel cilia-associated protein, is disrupted in the cpk mouse model of polycystic kidney disease. *J Clin Invest* 109:533–540, 2002
13. Lu W, Peissel B, Babakhanlou H, *et al*: Perinatal lethality with kidney and pancreas defects in mice with a targetted Pkd1 mutation. *Nat Genet* 17:179–181, 1997
14. Wu G, D'Agati V, Cai Y, *et al*: Somatic inactivation of Pkd2 results in polycystic kidney disease. *Cell* 93:177–188, 1998
15. Lu W, Fan X, Basora N, *et al*: Late onset of renal and hepatic cysts in Pkd1-targeted heterozygotes. *Nat Genet* 21:160–161, 1999
16. Kim K, Drummond I, Ibraghimov-Beskrovnaya O, *et al*: Polycystin 1 is required for the structural integrity of blood vessels. *Proc Natl Acad Sci USA* 97:1731–1736, 2000
17. Boulter C, Mulroy S, Webb S, *et al*: Cardiovascular, skeletal, and renal defects in mice with a targeted disruption of the Pkd1 gene. *Proc Natl Acad Sci USA* 98:12174–12179, 2001
18. Yoder BK, Hou X, Guay-Woodford LM: The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia. *J Am Soc Nephrol* 13:2508–2516, 2002
19. Lin F, Hiesberger T, Cordes K, *et al*: Kidney-specific inactivation of the KIF3A subunit of kinesin-II inhibits renal ciliogenesis and produces polycystic kidney disease. *Proc Natl Acad Sci USA* 100:5286–5291, 2003
20. Vanden Heuvel GB, Bodmer R, McConnell KR, *et al*: Expression of a cut-related homeobox gene in developing and polycystic mouse kidney. *Kidney Int* 50:453–461, 1996
21. Coqueret O, Berube G, Nepveu A: The mammalian Cut homeodomain protein functions as a cell-cycle-dependent transcriptional repressor which downmodulates p21WAF1/CIP1/SDI1 in S phase. *EMBO J* 17:4680–4694, 1998
22. Ledford AW, Brantley JG, Kemeny G, *et al*: Deregulated expression of the homeobox gene Cux-1 in transgenic mice results in downregulation of p27 (kip1) expression during nephrogenesis, glomerular abnormalities, and multiorgan hyperplasia. *Dev Biol* 245:157–171, 2002
23. Fero ML, Rivkin M, Tasch M, *et al*: A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* 85:733–744, 1996
24. Kiyokawa H, Kineman RD, Manova-Todorova KO, *et al*: Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* 85:721–732, 1996
25. Nakayama K, Ishida N, Shirane M, *et al*: Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 85:707–720, 1996
26. Combs HL, Shankland SJ, Setzer SV, *et al*: Expression of the cyclin kinase inhibitor, $p27^{kip1}$, in developing and mature human kidney. *Kidney Int* 53:892–896, 1998
27. Bhunia AK, Piontek K, Boletta A, *et al*: PKD1 induces p21(waf1) and regulation of the cell cycle via direct activation of the JAK-STAT signaling pathway in a process requiring PKD2. *Cell* 109:157–168, 2002
28. Lu W, Shen X, Pavlova A, *et al*: Comparison of Pkd1-targeted mutants reveals that loss of polycystin-1 causes cystogenesis and bone defects. *Hum Mol Genet* 10:2385–2396, 2001
29. Vanden Heuvel GB, Quaggin SE, Igarashi P: A unique variant of a homeobox gene related to Drosophila cut is expressed in mouse testis. *Biol Reprod* 55:731–739, 1996
30. Brantley JG, Sharma M, Alcalay NI, Vanden Heuvel GB: Cux-1 transgenic mice develop glomerulosclerosis and interstitial fibrosis. *Kidney Int* 63:1240–1248, 2003
31. Deng C, Zhang P, Harper JW, *et al*: Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G_1 checkpoint control. *Cell* 82:675–684, 1995
32. Park SK, Kim W, Lee H, *et al*: Differential changes of cell cycle regulators and activities in kidneys during pre- and postnatal development. *Nephron* 81:334–341, 1999
33. Lanoix J, D'Agati V, Szabolcs M, Trudel M: Dysregulation of cellular proliferation and apoptosis mediates human autosomal dominant polycystic kidney disease (ADPKD). *Oncogene* 13:1153–1160, 1996
34. Woo D: Apoptosis and loss of renal tissue in polycystic kidney diseases. *N Engl J Med* 333:18–25, 1995
35. Winyard PJ, Nauta J, Lirenman DS, *et al*: Deregulation of cell survival in cystic and dysplastic renal development. *Kidney Int* 49:135–146, 1996
36. Gattone VH, Ricker JL, Trambaugh CM, Klein RM: Multiorgan mRNA misexpression in murine autosomal recessive polycystic kidney disease. *Kidney Int* 62:1560–1569, 2002